# Optimization of the spontaneous tail coiling test for fast assessment of neurotoxic effects in the zebrafish embryo using an automated workflow in KNIME®

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# Abstract

Neuroactive chemicals are frequently detected in the environment. At sufficiently high concentrations or within mixtures, they could provoke neurotoxic effects and neurological diseases to organisms and humans. Fast identification of such neuroactive compounds in the environment could help in hazard assessment and risk mitigation. Behavior change is considered as an important endpoint and might be directly or indirectly connected to a neuroactive mode of action. For a fast evaluation of environmental samples and pure substances, we optimized the measurement of a behavioral endpoint in zebrafish embryos - the spontaneous tail coiling (STC). Evaluation of results is automated via the use of a workflow established with the KNIME® software. Analysis duration and developmental stage were optimized to 1 minute and 25 ± 1 hpf respectively during measurement. Exposing the embryos in a group of 10 or 20 and acclimatizing for 30 min at room temperature proved to be reliable. The optimized method was used to investigate neurotoxic effects of 18 substances with different modes of action (MoA). The STC test accurately detected the effect of 8 out of 11 neuroactive substances (chlorpyrifos, chlorpyrifos-oxon, diazinon, paraoxon-methyl, abamectin, carbamazepine, propafenone and diazepam). Aldicarb and nicotine showed subtle effects which were considered to be conditional and imidacloprid showed no effect. For substances with unknown neuroactive MoA, 3 substances did not provoke any effect on the STC (pyraclostrobin, diuron and daunorubicin-hydrochloride) while 4 other substances provoked an increased STC (hexaconazole, aniline, dimethyl-sulfoxide and 3,4-dichloroaniline). Such unexpected effects indicate possible neuroactive side effects or unknown mechanisms of action that impact on the STC. In conclusion, the optimized STC parameters and the automated analysis in KNIME® indicate opportunities for the harmonization of the STC test and further development for prospective and diagnostic testing.

Keywords: Acetylcholinesterase inhibitors; Developmental neurotoxicity; Behavioral toxicology; Spontaneous activity; Hyperactivity; Alternatives to animal testing

# 1. Introduction

Neuroactive substances are frequently detected in the environment and environmental concentrations may induce adverse effects such as neurological damage in humans and in the ecosystem (Busch et al. 2016). To prevent neurotoxic hazard, it is necessary to develop new, fast and sensitive toxicological tests to screen neuroactive substances. Behavior tests such as locomotor activity are considered to be sensitive and specific to detect neurotoxic effects since it is anticipated that behavior is directly or indirectly related to the function of the nervous system. Such behavior tests have been utilized for both drug development and toxicity testing in animals such as rodents, fish and amphibians (OECD 2007a; OECD 2007b; Parker 2016; Tierney 2011). However, alternative techniques are required to reduce the time, cost and number of animals in developmental neurotoxicity testing (Bal-Price et al. 2015). Currently early life stages of fish are particularly gaining wide acceptance for use in behavior testing due to the non-protection of these stages as well as possibility for small-scale and high throughput testing (Basnet et al. 2019; Braunbeck et al. 2005; Legradi et al. 2015; Ogungbemi et al. 2019; Scholz et al. 2013).

In particular, zebrafish embryos represent an attractive toxicity testing model for several reasons: its small size allows the use of low quantity exposure solution, its fast development makes it amenable to short duration testing and its transparency enables the assessment of developmental effects and protocols for the assessment of early behavioral features such as spontaneous tail coiling are available (Hill et al. 2005; Scholz et al. 2013). Furthermore, due to the conservation of principal mechanisms of neurotoxicity in animals, testing of zebrafish embryos also allows extrapolation to other species including humans. A previous review of different zebrafish embryo behavior tests (Ogungbemi et al. 2019) had indicated that

the spontaneous tail coiling (STC) of zebrafish embryos could represent a reliable endpoint to detect neurotoxicity and hence this endpoint was selected for further optimization in the present study. The STC consists of single or alternating tail coilings which can be observed as early as 19 hours post fertilization (hpf) in the developing embryo (Kimmel et al.1974; Saint-Amant and Drapeau 1998). The observed tail coilings are assumed to occur as a result of innervation of the muscle by the primary motor neurons and therefore, measurement of the STC frequency could be a good indicator of adverse effects to the function and development of the muscle innervation or generally the nervous system.

In previous studies, the STC test has been used to analyze effects of neuroactive chemicals such as abamectin, chlorpyrifos, carbamazepine etc. (Cheng et al. 2017; Selderslaghs et al. 2010; Vliet et al. 2017; Weichert et al. 2017). STC response of these chemicals relative to negative control appears to be a promising technique to predict either the stimulatory or inhibitory mode of action (MoA) of neuroactive compounds (Ogungbemi et al. 2019). For example, the hyperactivity (referring to increased STC) effect of chlorpyrifos-oxon may be correlated to its stimulatory action when it inhibits acetylcholinesterase enzyme while the hypoactivity (decreased STC) effect of abamectin may be linked to its inhibitory action when it activates Gamma aminobutyric acid (GABA) receptors (Raftery and Volz 2015).

However, reports on the use of the STC test method vary in their experimental protocol and how effects are estimated. This may lead to lack of reproducibility and usability of results for the identification of specifically acting neuroactive substances. For instance, differences in effect concentration for abamectin may be attributed to the use of different exposure material (Ogungbemi et al. 2019; Raftery et al. 2014); different effect concentrations for dichlorvos may relate to the use of different endpoint – frequency or duration of STC (Watson et al. 2014; Zindler et al. 2019); inconsistent effects (hyper- or hypoactivity) were also reported for paraoxon and this could be attributed to estimating the endpoint in different ways –

percentage of embryos showing STC versus frequency of STC (Ogungbemi et al. 2019; Yozzo et al. 2013); and different substances were indicated as potential neurotoxic depending on a short (2 h) or a long (23 h) exposure duration (Vliet et al. 2017). Other experimental parameters such as age or developmental stage of embryo, duration of behavioral analysis and sample size could influence the STC result leading to incoherent interpretations. Richendrfer et al. (2014) also showed that variation in the age of embryo in reported STC studies could influence behavioral analysis. Hence, there is a need to optimize these experimental parameters for appropriate interpretation of neurotoxicity. Crofton et al. (2011) suggests a list of guidelines to develop alternative test methods for developmental neurotoxicity testing. These recommendations could also facilitate validation of the STC test for the use in hazard assessment and effect-based environmental monitoring.

The aim of the present study was to investigate the influence of experimental parameters on the STC response and to develop an optimized STC test for screening neuroactive compounds. We optimized important experimental parameters and created an automated workflow to measure the STC in the open access software KNIME® (Berthold et al. 2009). Subsequently, we implemented the guidelines recommended by Crofton et al. (2011) to establish an optimized STC protocol. We tested the new protocol on 18 chemicals with different modes of action - either with an expected activation or inhibition of movement or without any expected effect.

# 2. Materials and Method

## 2.1 Test organism

Fish cultivation, feeding and embryo collection was conducted as described previously (Massei et al. 2015). Briefly, two strains of adult zebrafish (OBI and WIK strains) were crossed to produce a hybrid strain (OBI-WIK strain, F3 generation) in order to avoid inbred effects. The strain was cultured under 14h

light/10h dark photoperiod in 120 L aquaria (tap water, 26.5±1 °C). Spawning trays were inserted on the afternoon 4-6 hours before the end of the light cycle. To initiate spawning, lights were automatically switched on at 8am the following day and eggs were collected at 9am inside a rectangular glass dish covered with a stainless steel sieve. Fertilized and normal embryos were selected according to Kimmel et al. (1995) with a binocular microscope and embryos between 16 and 128 cell stage were used for the experiments.

## 2.2 Media and Chemicals

Information about the purity and manufacturer of all chemicals are shown in SI Table S1. Stock solutions were prepared either in ISO water as specified in ISO 7346-3 (1996) [80 mM $CaCl_2 \cdot 2H_2O$, 20 mM $MgSO_4 \cdot 7H_2O$, 31 mM $NaHCO_3$, 3.1 mM KCl] or in 100 % dimethyl-sulfoxide (DMSO). All chemicals were dissolved in DMSO except; imidacloprid, 3,4-dichloroaniline, aniline, daunorubicin-hydrochloride, diazinon and nicotine. For preparation in ISO water, test chemicals (except liquid substances) were prepared a day before exposure and left to stir overnight for dissolution. The DMSO stock solutions were diluted to lower concentrations in ISO water during exposure and the DMSO concentrations varied along the dilution series but never exceeded 0.1% (v/v) in diluted solutions.

## 2.3 Chemical exposures

The chemicals were grouped by their expected effects in the STC in relation to their known mode of action: chlorpyrifos, chlorpyrifos-oxon, diazinon, paraoxon-methyl, aldicarb, imidacloprid and nicotine were anticipated to represent hyperactive chemicals; abamectin, carbamazepine, diazepam and propafenone were considered to represent hypoactive chemicals; chemicals with unknown neuroactive mode of action or without any expected effect to the STC were represented by diuron, aniline,

pyraclostrobin, hexaconazole, daunorubicin-hydrochloride, DMSO and 3,4-dichloroaniline. Exposure concentrations are given in SI Table S1 and these were selected based on mortality data from published literature or in-house unpublished mortality data. Briefly, twenty fertilized embryos (1-3 hpf) were exposed in 20 mL of diluted stock solution or ISO water as control, within a 60 mm glass crystallization dish covered with a watchmaker glass. A solvent control was used when the substance was dissolved in DMSO. The exposed embryos were incubated at 28°C under 14h light/10h dark photoperiod for 21 ± 1h. The exposure was conducted using 2 technical parallel replicates and at least 2 independent replicates to get sufficient amount of data for the concentration-response modelling. pH of the highest concentration and control solution were measured before and after the experiment to control for possible changes within the exposure time.

### 2.4 Measurement of the spontaneous tail coiling (STC)

At 24 hpf, exposed embryos were removed from the incubator and allowed to acclimatize to room temperature for at least 30 min. Embryos were inspected for lethality/malformations and affected embryos were separated. Samples with less than 20% affected embryos were considered valid for STC assessment. Videos of normally developed embryos were recorded for 60 s (frame rate of 2 frames per second) with a video camera (Olympus DP21, Hamburg, Germany) mounted to an Olympus SZX7 stereomicroscope (0.8x magnification). The embryos were recorded in groups of 20 using a black background and dark field transmitted light at the base of the microscope, with an ISO speed of 400, time of exposure of 1/80 and image size of 400x300 pixels. Collected videos were analyzed for STC counts by means of a workflow using the KNIME® Analytical Platform (Berthold et al. 2009). Occasionally two tail coilings appear very close together. In such cases the camera setting of 2 frames per second could not resolve them as individual coilings, these were counted manually.

## 2.5 Influence of experimental parameters

### 2.5.1 **Exposure duration** and developmental stage of analysis

To investigate the optimal exposure duration or developmental stage during behavior analysis for zebrafish embryos in the STC test, 20 embryos (<3hpf) were exposed in ISO water and STC was measured hourly starting from 21 hpf to 31 hpf. This experiment was conducted with 3 technical replicates.

### 2.5.2 Acclimation duration

STC measurement and video recording were not undertaken in temperature controlled chambers. As a result, to investigate the influence of temperature changes during acclimation time (after removal from the incubator at 28$^{o}$C and before STC measurement) on the STC response, 20 embryos per treatment were exposed in ISO water and temperature was measured during acclimation. Treatment 1 = control (no acclimation); Treatment 2 = 15 minutes acclimation at room temperature; Treatment 3 = 30 minutes acclimation at room temperature. After incubation, treatment 2 and 3 were removed from the incubator at 15 and 30 minutes respectively before STC measurement. Treatment 1 was not acclimatized under room temperature but measured as immediately possible. Three technical replicates were used for all treatments and STC measurement was conducted between 24 – 25 hpf for all embryos.

### 2.5.3 Sample size

To evaluate the effect of simultaneously reducing sample size (20 to 10 embryos per replicate) and increasing the number of replications (3 to 5 replicates) on variability of the STC response, two treatments were considered. In the first treatment, 20 embryos of 3 replicates were exposed in ISO water. 10 embryos of 5 replicates were used in the second treatment. The experiment was repeated thrice and STC measurement was conducted between 24 – 25 hpf. Additionally, already collected and analyzed STC control data were reanalyzed by estimating the mean of 10 embryos in comparison to the mean of 20 embryos per sample.

### 2.5.4 Analysis duration

The impact of reducing the analysis duration of the STC was investigated. STC data for abamectin and chlorpyrifos were re-analyzed in the KNIME® workflow in which the recorded video of 60s was segmented into different time bins of 60, 30, 20 and 10s.

### 2.5.5 Rearing condition

To test if the movement of one embryo might stimulate the movement of other nearby embryos and therefore accidentally influence outcome, we reared embryos with ISO water in single or group conditions. In single condition, 10 embryos were individually placed in 10 glass crystallization dishes and in 2 replicates (one embryo per dish per 10 dishes and a total of 20 dishes). Group condition was implemented by placing 10 embryos in a group within the same dish (10 embryos per dish and 2 replicates per dish). STC measurement was conducted between 24 – 25 hpf.

## 2.6 Image analysis parameters

To optimize the image analysis of STC in KNIME®, we investigated the influence of parameters like threshold (thrs) and the so called smoothing parameter (spar) used for identification of peaks within the R-snippet node in KNIME®. Threshold is the value beyond which the STC counts as one. Any response below this value was attributed to noise. The higher the threshold, the lower the sensitivity. Smoothing parameter is responsible for the smoothing of the response peak signal. Smoothing removes small peaks assumed to represent signal noise. The higher the smoothing parameter, the lower the peak signal, and hence the lower the sensitivity to detect small peaks or the higher the possibility that smaller peaks will be counted as noise. These parameters were manipulated or changed in an R script (function smooth.spline and test peaks within dcpR package) embedded in KNIME®. Manipulated threshold values were - 0.001, 0.002, 0.003, 0.004 and 0.005 while smoothing parameter values – of 0.1, 0.2 and 0.3 were applied. The analysis was done by varying the threshold parameter for each level of the smoothing

parameter. Three independent experiments were conducted for untreated embryos. The resulting STC response in KNIME® was then compared to a manual STC count.

### 2.7 Data analysis

STC was expressed as the number of STCs per minute (frequency) for one embryo. The mean STC frequency was estimated for a group of 20 embryos that were subject to the same treatment. The absolute STC frequency varied between the independent experiments while the trend provoked by treatments was conserved. To combine results from independent experiments, a normalized percentage mean STC frequency was obtained by dividing the mean STC frequency by the respective mean STC frequency for control embryos and multiplying by 100. Data for hypoactivity modeling were further treated by adding 100 to convert the negative values to positive. Concentration-response modeling of the percentage STC frequency was performed using the 4-parameter logistic function (LL.4) of the drc package in R (Ritz and Streibig 2005).

$$y = c + \frac{(d - c)}{1 + (\frac{x}{e})^b}$$

Where b is the slope function; c and d are the minimum and maximum STC response respectively; and e is the $EC_{50}$.

In cases of hyperactivity, the maximum parameter d in the model was fixed as the highest hyperactivity response. The effect concentration causing 10 and 50% increase or decrease of the STC was estimated from the concentration-response curve. Some compounds showed biphasic response (i.e. initial hyperactivity and declining hypoactive response at higher concentrations). The hypoactivity at higher concentration could be a result of strong seizures due to over-excitation or represent a result of subtle

malformation and overt toxicity (Behra et al. 2002; Stehr et al. 2006). Hence, these data were not included in constructing concentration-response models. Hypothesis testing was used to check for differences in experimental parameters. Shapiro test and Bartlett test were used to check for normality and homogeneity of variance, respectively. Analysis of variance or Friedman test were used to test for statistical differences between treatment groups. Bonferroni adjusted Wilcoxon signed-rank test was used as a post-hoc test. Statistical difference was considered when the p-value < 0.05. Sensitivity ratio (SR) was calculated by dividing the available $LC_{50}$ data with the STC $EC_{50}$ data (Bittner et al. 2019). SR > 1 means the STC $EC_{50}$ is more sensitive than $LC_{50}$ i.e. STC effect is observed at a factor (factor of SR) lower concentrations than lethal effect and vice versa when SR < 1. Low SRs close to 1 indicate that the effect on STC was observed close to mortality.

# 3. Results

## 3.1 Influence of experimental parameters

The spontaneous tail coiling (STC) frequency depends on the developmental stage used for the assessment. This has been reported previously (Cheng et al. 2017; Saint-Amant and Drapeau 1998) and was confirmed for our experimental setup. A weak STC frequency (1 count per minute) was observed at 21 and 22 hpf, with maximum values (3.5 counts per minute) at 23 and 24 hpf, followed by a gradual decline until 31 hpf (Figure 1). Acclimation duration does not affect the STC response when acclimation under room temperature is ≤ 30 minutes. After removal of the exposure dish from the incubator (28°C), the measured temperature of the solution was ≈ 25°C and this declined to a stable value of 22.8°C after 30 minutes acclimation under room temperature (SI Table S2 and S3). There were no statistical differences (p-value = 0.542) in STC response between control (no acclimation), 15 minutes acclimation and 30 minutes acclimation. Sample size manipulation did not seem to affect the variability of the STC after

reducing the number of embryos in a dish from 20 to 10, and simultaneously increasing the number of replicates from 3 to 5. The means and standard deviations of the different setups were similar (SI Table S4). Additionally, analyzing a sample size of 10 and 20 embryos from the same dish resulted in no observable differences (SI Figure S1). Single or group rearing conditions did not seem to influence the STC response. A comparison of standard deviations shows there is no difference between both setups and this suggests that group exposure does not probably cause contagious stimulation of STC in neighboring embryos (SI Figure S2). To evaluate the influence of analysis duration on STC response, we selected typical hyperactive (chlorpyrifos) and hypoactive (abamectin) substances. Comparing the STC frequency for different analysis duration of 60, 30, 20 and 10 s shows a slightly declining STC trend from 60 to 10 s in all the dataset considered (Figure 2). However, this decline was not statistically significant.

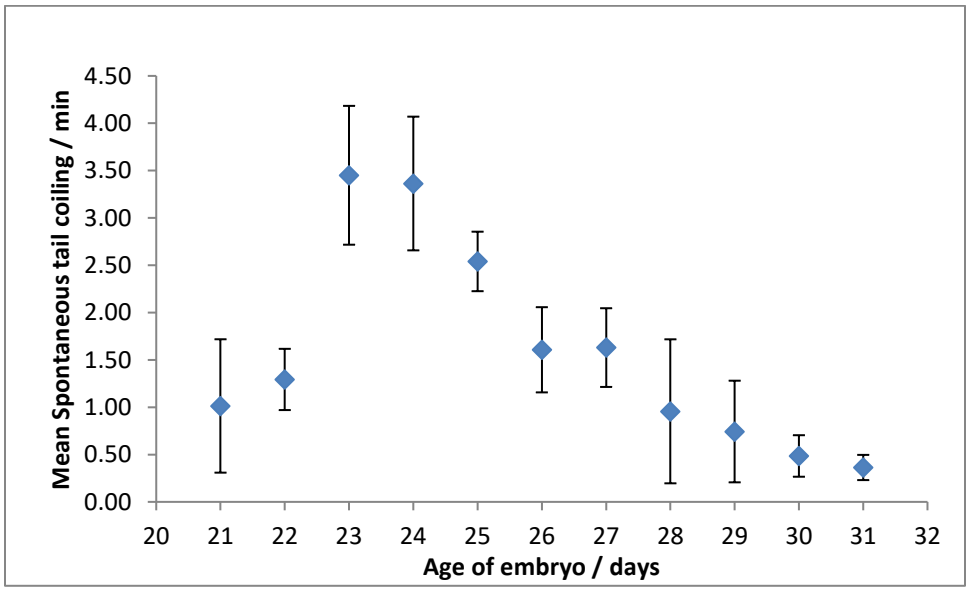



Figure 1: Effect of exposure duration (or developmental stage) on STC response for untreated embryos. Embryos were incubated at 2hpf at 28°C and monitored at 21hpf hourly till 31hpf. Twenty embryos were measured per replicate. Data points show mean value of 3 replicates and error bars represent standard deviation.

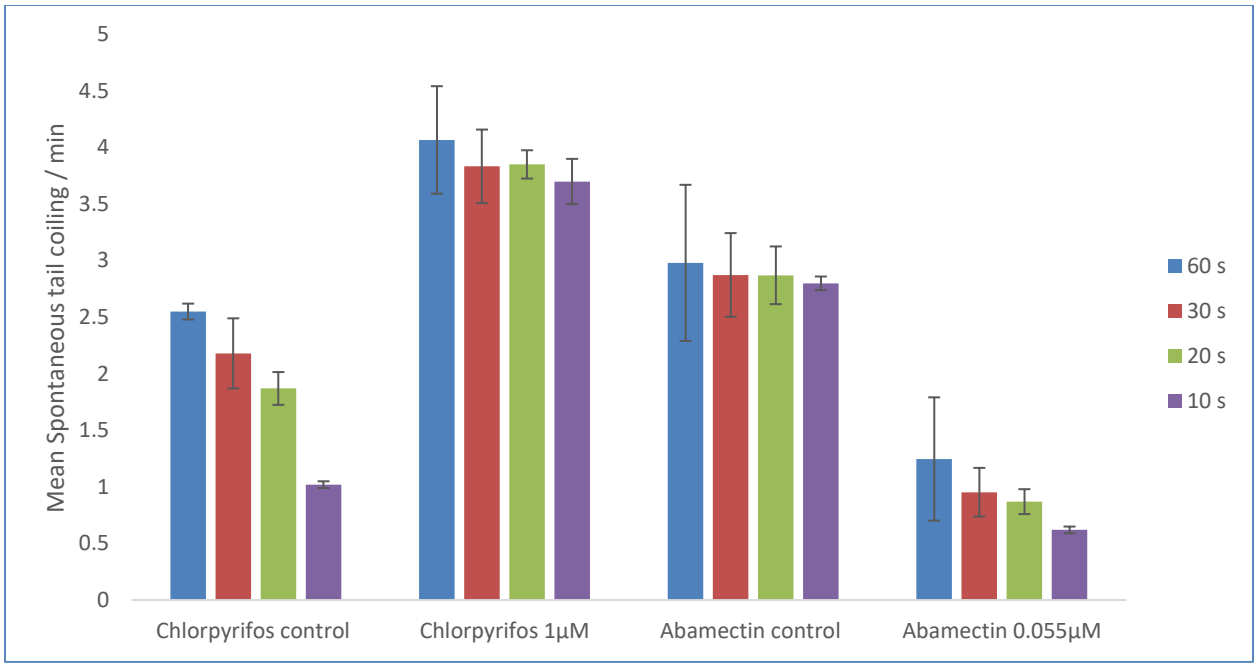
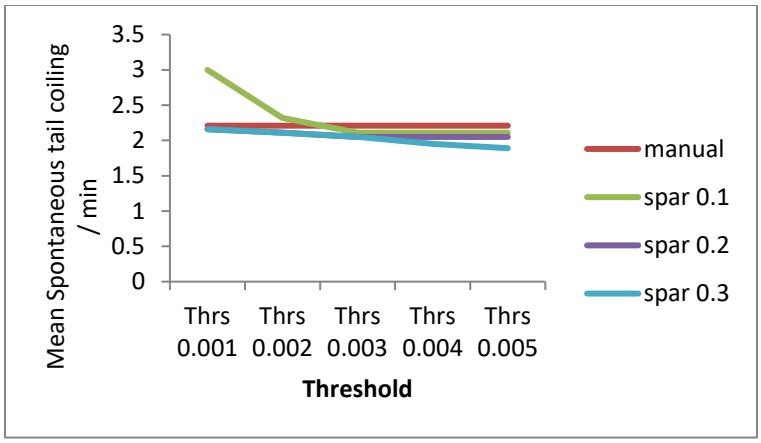



Figure 2: Comparison of STC frequency from different analysis duration of 60, 30, 20 and 10s. Analysis was done for chlorpyrifos and abamectin at specific concentrations showing effect on the STC. Chlorpyrifos control and abamectin control refer to DMSO solvent control. Data points show mean value of 3 replicates and error bars represent standard deviation. A Friedman test showed no statistical significant difference (p-values of 0.042, 0.72, 0.80, 0.085) between the analysis duration of each treatment (chlorpyrifos control and 1um; abamectin control and 0.055 respectively). A further Wilcoxon sign-rank post-hoc test for chlorpyrifos control showed no statistical significance.

A)

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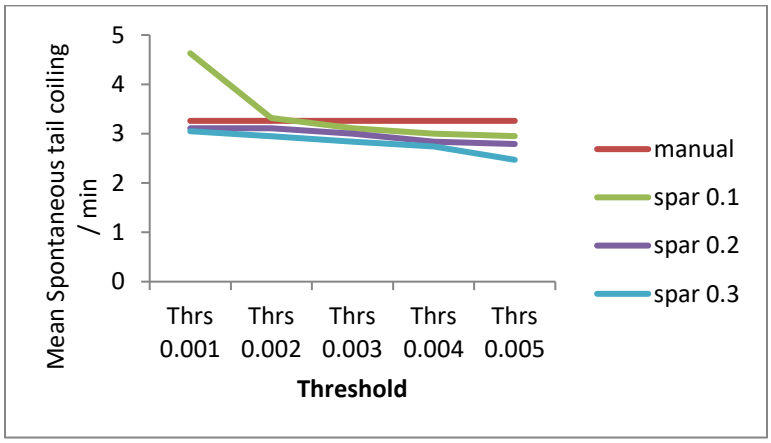
284 B)

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286 C)

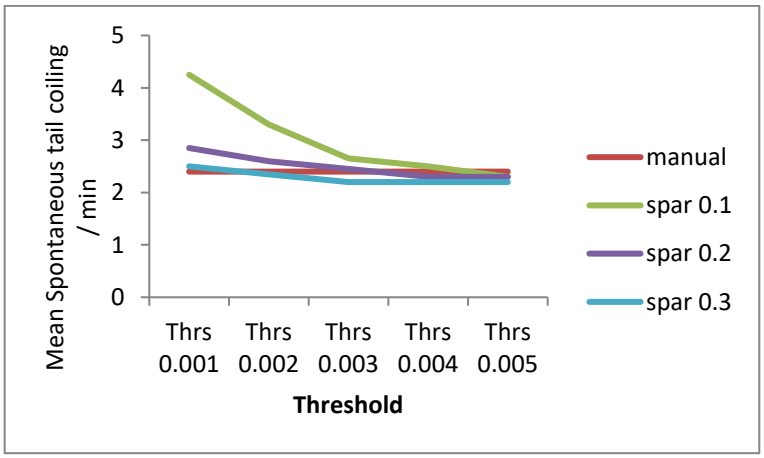


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Figure 3: Effect of threshold (thrs) and smoothing parameter (spar) for comparison of results to manual counting of STCs are shown. A, B and C represent 3 independent experiments. Increase in threshold or spar leads to a decrease in the STC response. For subsequent analysis, parameters were selected that resulted in highest concordance between manual and automated assessment of STC frequency.

### 3.2 Influence of image analysis parameters

Two parameters used for image analysis namely threshold (thrs) and smoothing-parameter (spar) can particularly influence the calculation of STC counts in the KNIME® workflow. The comparison of different threshold and smoothing parameters show an inverse relationship between STC response and threshold or smoothing-parameter (Figure 3). This trend was most obvious for the smoothing parameter of 0.1. To obtain optimal parameter setting with results similar to manual STC count, smoothing- and threshold were selected as 0.0025thrs/0.1spar, 0.002thrs/0.1spar and 0.0035thrs/0.2spar for the 3 independent replicates respectively. Based on visual observation of the graphs (Figure 3) we selected 0.003thrs/0.1spar parameters for all subsequent analysis given that these parameters were showing the highest concordance with manual analysis.

### 3.3 Effect of chemicals in the STC test

Effect concentrations of all chemicals are reported in Table 1. Observed STC effects for each chemical were compared to the expected effect based on the chemical's mode of action. Among chemicals which are expected to cause hyperactivity; diazinon, chlorpyrifos, chlorpyrifos-oxon and paraoxon-methyl displayed a clear hyperactivity response with $EC_{50}$s of 5.24, 1.85, 0.32 and 4.13 µM respectively (Figure 4). Additionally, the hyperactivity for chlorpyrifos-oxon peaked at 1 µM and started to decline at 1.76 µM. Diazinon caused up to 50% mortality at 10 µM while paraoxon-methyl at 100 µM caused sublethal effects

such as incomplete tail coiling and reduced-resorption of the yolk sac (SI Figure S3). Nicotine and aldicarb also showed subtle hyperactivity at $EC_{50}$s of 0.97 and 29.6 µM respectively (Figure 4). However these hyperactivity effects were not consistent and highly variable, hence we considered them as conditional effects. To test the influence of exposure duration as an explanation for lack of clear nicotine effect, embryos were exposed to nicotine for 20 mins between 24-25hpf. In contrast to the longer duration exposure in which only mild effects were observed, nicotine induced clear hyperactivity in all tested concentrations of 10, 20, 30, 40 µM (SI Figure S4). Imidacloprid showed no effect in the STC test up to 2000 µM.

Among chemicals which are expected to cause hypoactivity; abamectin, carbamazepine, diazepam and propafenone all caused hypoactivity with $EC_{50}$s of 0.055, 271, 20.9 and 31.6 µM respectively (Figure 4). Additionally, diazepam at 50 and 100 µM induced sublethal effects such as reduced-resorption of the yolk sac and oedema of the pericard.

In search for negative control substances, different chemicals which do not have a known neuroactive mode of action were tested. Diuron, an herbicide, showed no significant effect up to 8 µM and caused 100% mortality at 16 µM. Daunorubicin-hydrochloride, an antimitotic drug showed no STC effect up to 50 µM. Pyraclostrobin, a fungicide showed no STC effect up to 0.14 µM (SI Figure S5). Higher concentrations of 0.2 and 0.25 µM caused sublethal effects, such as reduced-resorption of the yolk sac, no tail detachment and no clear formation of the head, which could be indications of developmental delay (SI Figure S3), while 0.4 µM caused between 50 – 100% mortality. Aniline, a known baseline toxic/narcotic substance caused hyperactivity at $EC_{50}$ of 832 µM while 3000 µM induced 100% mortality. 3,4-dichloroaniline, a precursor and metabolite of diuron also caused hyperactivity at $EC_{50}$ of 5.79 µM. Hexaconazole, a fungicide, caused hyperactivity ($EC_{50}$ = 4.03 µM) up to a maximum concentration of 15 µM and higher concentration of 25 µM caused a decline of the activity towards control level (Figure 4). DMSO, a commonly used solvent induced hyperactivity at $EC_{50}$ of 275455 µM (1.96%).

337 Table 1: Summary of STC effect characterization for all chemicals exposed to zebrafish embryos. Data

338 collected in the present study are effect concentrations and confidence intervals (In parenthesis).

339 Expected activity was inferred from the mode of action of each chemical

| **Substance** | **Mode of Action**[m] | **Expected activity** | **Observed activity** | **STC $EC_{10}$ (µM)** | **STC $EC_{50}$ (µM)** | **0-48 hpf $LC_{50}$ (µM)** | **Baseline toxicity**[t] **(µM)** | **Sensitivity Ratio $LC_{50}/EC_{50}$** |
|---|---|---|---|---|---|---|---|---|
| **Chlorpyrifos** | Acetylcholinesterase inhibitor | Hyperactivity | Hyperactivity | 0.35 (0.11-0.59) | 1.85 (1.37-2.33) | 5.4[+d] | 1.85 | 2.9 |
| **Chlorpyrifos oxon** | Acetylcholinesterase inhibitor | Hyperactivity | Hyperactivity | 0.047 (0.003-0.09) | 0.32 (0.2-0.43) | 1.5[w] | 54.1 | 4.7 |
| **Diazinon** | Acetylcholinesterase inhibitor | Hyperactivity | Hyperactivity | 3.46 (2.3-4.6) | 5.24 (4.58-5.9) | 19.7[d] | 17.7 | 3.7 |
| **Paraoxon-methyl** | Acetylcholinesterase inhibitor | Hyperactivity | Hyperactivity | 0.81 (-2.12-3.74) | 4.13 (1.36-6.9) | 230[d] | 1097 | 55.7 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Aldicarb** | Acetylcholinesterase inhibitor | Hyperactivity | Hyperactivity | - | 29.6[#] (-2.16-2.75) | 279.9[+k] | 7967 | 9.4 |
| **Nicotine** | Nicotinic acetylcholine receptor agonist | Hyperactivity | Hyperactivity | 0.69[#] (-1.79-3.19) | 0.97[#] (0.09-1.85) | 3353 [e] | 6792 | 3456 |
| **Imidacloprid** | Nicotinic acetylcholine receptor agonist | Hyperactivity | No effect | - | - | | 28556 | - |
| **Abamectin** | Activation of GABA-gated chloride channel; glutamate- | Hypoactivity | Hypoactivity | 0.015 (0.0039-0.026) | 0.055 (0.035-0.074) | 0.7 [w] | 4.61 | 12.7 |
| **Propafenone** | Sodium channel blocker | Hypoactivity | Hypoactivity | 9.5 (2.8-16.3) | 31.6 (23-40) | 81[d] | 45.1 | 2.56 |
| **Carbamazepine** | Sodium channel blocker | Hypoactivity | Hypoactivity | 104 (-0.99-209) | 271 (193-350) | 263 [d] | 393.1 | 0.97 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Diazepam** | GABA receptor agonist | Hypoactivity | Hypoactivity | 14.8 (6.4-23.2) | 20.9 (15.3-26.5) | | 169.1 | 8.1 |
| **Pyraclostrobin** | Respiration inhibitor | No activity | No effect | - | - | 0.26[*b] | 9.14 | - |
| **Diuron** | Photosystem II inhibitor | No activity | No effect | - | - | 12.6[*d] | 233 | - |
| **Aniline** | Narcosis | No activity | Hyperactivity | 736 (583-890) | 832 (734-930) | 1910[*b] | 8929 | 2.3 |
| **Daunorubicin HCl** | Topoisomerase II inhibitor | No activity | No effect | - | - | 110[e] | 2029 | - |
| **Hexaconazole** | Inhibits ergosterol biosynthesis | No activity | Hyperactivity | 1.18 (-0.106-2.47) | 4.03 (1.78-6.28) | 65[d] | 22.2 | 16 |
| **3,4 dichloroaniline** | Metabolite of diuron | No activity | Hyperactivity | 2.18 (-0.4-4.75) | 5.79 (2.53-9.05) | 15.2[*d] | 222.3 | 2.6 |
| **Dimethyl sulfoxide** | Solvent | No activity | Hyperactivity | 275455 (232094-318817) | 213851 (-83686-511389) | 454755[d] | 2272479 | 1.65 |

340

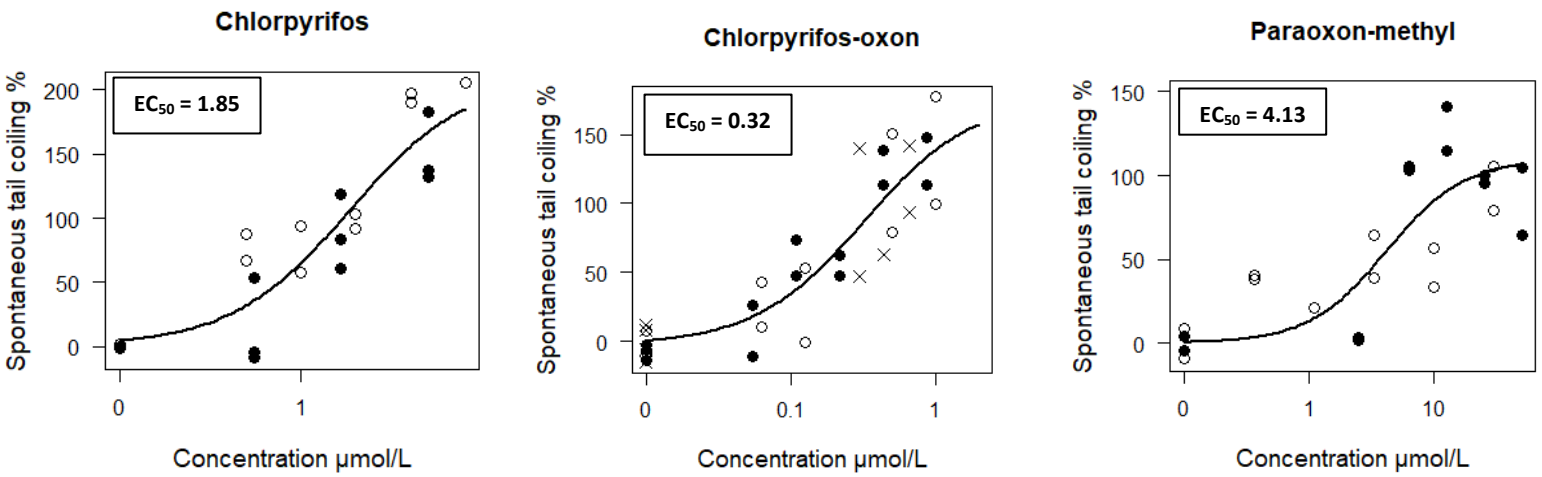
341 [#]Conditional effect due to inconsistency between replicates. *data for 0-24hpf. [+]data for 0-96hpf. [k]data

342 from Klüver et al 2015. [b]data from Birke and Scholz 2019. [d]unpublished data of the Helmholtz Centre for

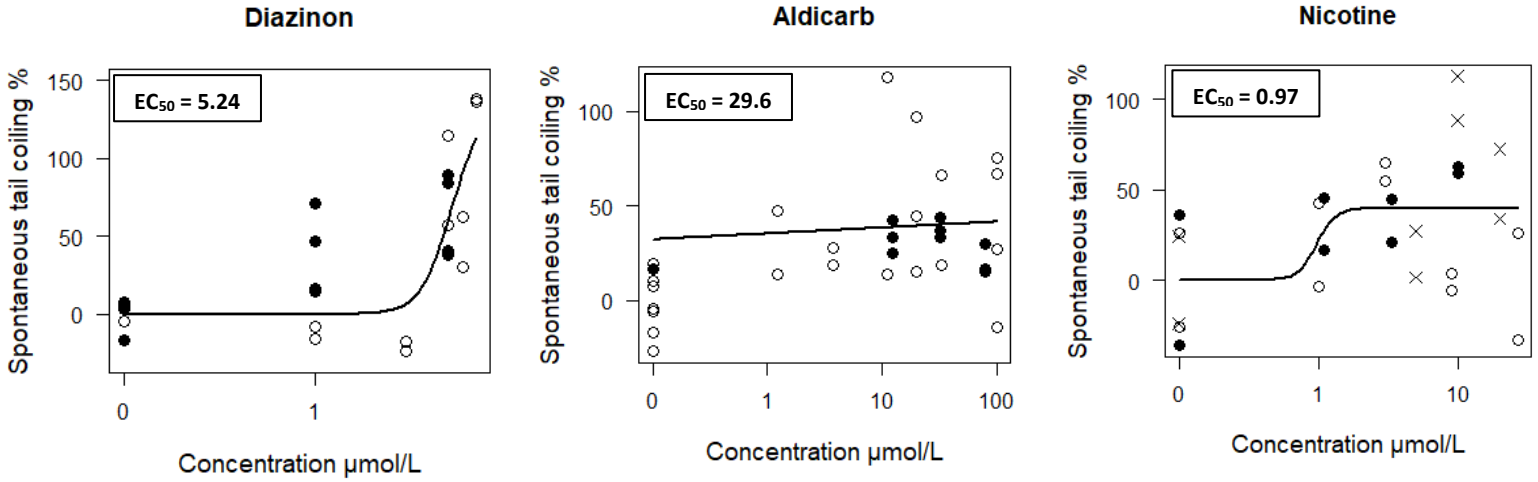
343 Environmental Research. [w]data from Weichert et al. 2017. [m]Mode of action was obtained from different

344 sources including http://drugbank.ca, pesticide properties database and published literature. [t]Baseline

345 toxicity is the lethal concentration predicted from lipophilicity estimated from Klüver et al. 2016.

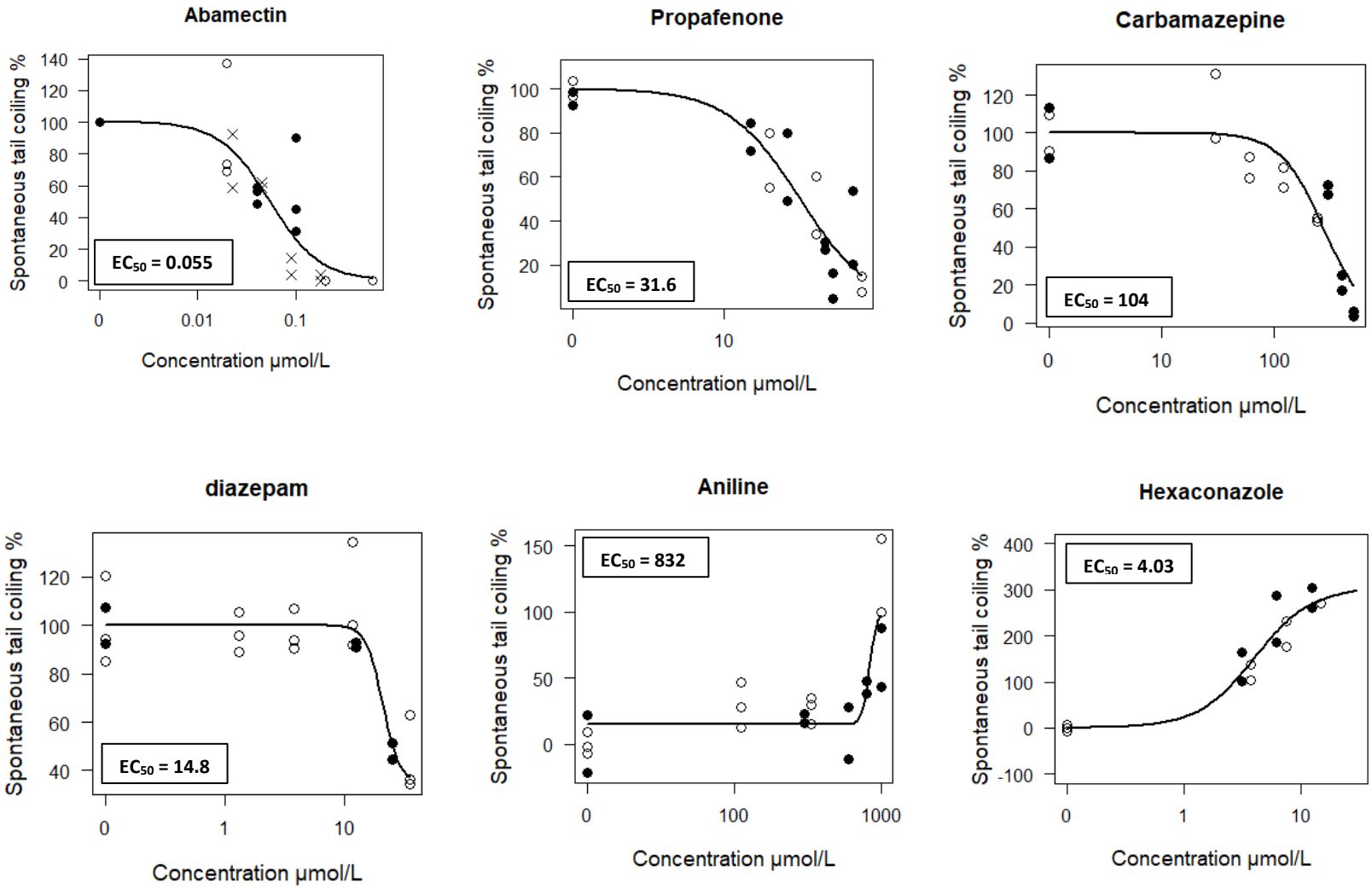

Chlorpyrifos
EC50 = 1.85
Spontaneous tail coiling %
Concentration µmol/L
Chlorpyrifos-oxon
EC50 = 0.32
Spontaneous tail coiling %
Concentration µmol/L
Paraoxon-methyl
EC50 = 4.13
Spontaneous tail coiling %
Concentration µmol/L

346


Diazinon
EC50 = 5.24
Spontaneous tail coiling %
Concentration µmol/L
Aldicarb
EC50 = 29.6
Spontaneous tail coiling %
Concentration µmol/L
Nicotine
EC50 = 0.97
Spontaneous tail coiling %
Concentration µmol/L

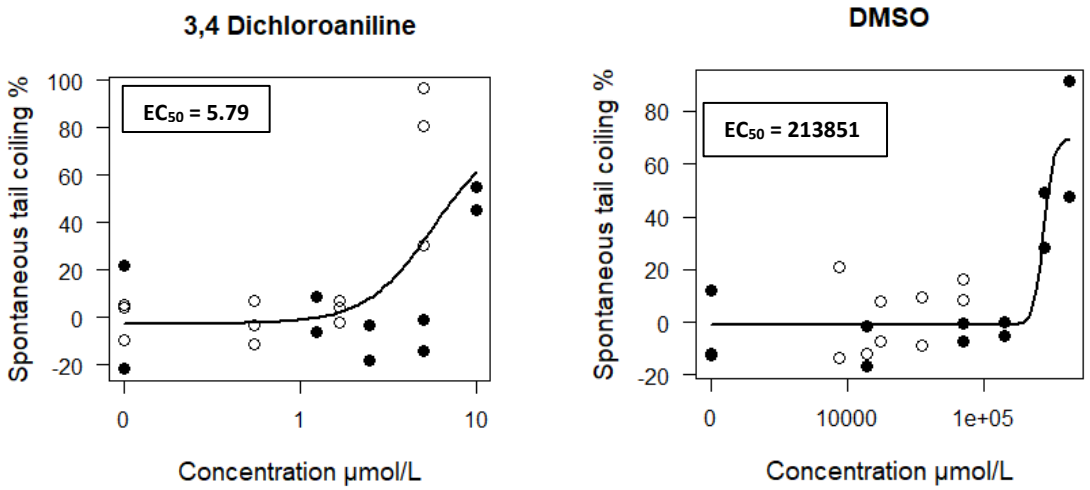
347

348


Abamectin
Spontaneous tail coiling %
EC50 = 0.055
Concentration µmol/L
Propafenone
Spontaneous tail coiling %
EC50 = 31.6
Concentration µmol/L
Carbamazepine
Spontaneous tail coiling %
EC50 = 104
Concentration µmol/L
diazepam
Spontaneous tail coiling %
EC50 = 14.8
Concentration µmol/L
Aniline
Spontaneous tail coiling %
EC50 = 832
Concentration µmol/L
Hexaconazole
Spontaneous tail coiling %
EC50 = 4.03
Concentration µmol/L

349

350




351

352

353 Figure 4: Concentration-response curves for chemicals impacting on the frequency of spontaneous tail
354 coiling. Y-axis represents spontaneous tail coiling normalized to control and X-axis shows the exposure
355 concentration. Different symbols represent independent experiments. Upward curves indicate
356 hyperactivity effect with respect to controls while downward curves indicate hypoactivity effect.

357

358

359

# 4. Discussion

Screening and detection of neuroactive substances is a major challenge in environmental protection. Assessment of animal behavior as an integrative endpoint appears to be a very promising approach to screen for compounds with diverse neuroactive mode of actions. Infact, zebrafish embryo behavior tests are considered to fill the gap for the probable insufficient capacity of the fish embryo test (FET) to screen neuroactive compounds (Sobanska et al. 2018; Klüver et al. 2015). However, systematic assessment of the predictivity and reliability of behavior endpoints are lacking. Available behavioral methods such as the locomotor response test, spontaneous tail coiling test and photomotor response test (reviewed in Ogungbemi et al. 2019) are either not sufficiently specific to detect only neuroactive substances or they are restricted in their diagnostic capacity to detect a wide range of neuroactive substances. Behavior tests used in regulation also require conduction of experiments with adult animals which are subject to ethical concern and are cost- and labor-intensive (OECD 2007a and b). To exploit alternatives to animal testing, we explored the reliability of the STC test as an alternative screening system for the detection of (developmental) neurotoxic compounds. The STC test represents one of the available fish embryo behavior tests and has been proposed to detect chemicals interfering with motor neurons. However, a limited diagnostic capacity of the STC could occur because of 1.) Possible incapability to reveal responses in the brain due to effects being majorly propagated from the spinal cord; 2.) Possible limited biotransformation capacity of early stages of the embryo; 3.) Probable low internal concentration of chemicals that are slowly taken up (e.g. charged or hydrophobic compounds); and 4.) Possible limited uptake of high molecular weight substances due to the chorion (pore-size) barrier. At present, it is difficult to estimate the diagnostic capacity given that diverse protocols are used for STC assessment. Hence, it is necessary to characterize the extent of sensitivity and specificity of different test setups and associated parameters. Crofton et al. (2011) described a set of guidelines for developing and optimizing alternative tests for developmental neurotoxicity. We used these guidelines to characterize the capacity of the STC

384 test to detect neuroactive substances. In the present study, we assessed the influence of experimental
385 parameters on the variability and reproducibility of the STC response. An optimized experimental protocol
386 was then validated using 11 chemicals known to interact with the nervous system and 7 others which are
387 not primarily known to disrupt or affect the nervous system.
388
389 **4.1 Discussion of the STC test performance in relation to guidance for (developmental)**
390 **neurotoxicity testing**
391 **4.1.1 Key event of neurodevelopment -** Endpoints should model key aspects of neurodevelopment

392 Spontaneous tail coiling (STC) represents the first motor activity generated by the developing neural
393 network which occurs as a result of the innervation of the muscle and is assumed to support hatching of
394 the embryo from its chorion (Kimmel et al. 1974; Saint-Amant and Drapeau 1998). The STC is presumed
395 to be generated by depolarizations which trigger action potentials in the synapses of the primary motor
396 neurons (Drapeau et al. 2002). These synapses leading to STC are assumed to be mainly due to an
397 electrically coupled network in the spinal cord (Saint-Amant and Drapeau 2000). This raises uncertainties
398 about the contribution of chemical neurotransmitters to mediate the observed STC or if they are present
399 at this early stage of development. Tufi et al. (2016) measured different neurotransmitters including
400 acetylcholine and GABA in 24 hpf embryos and hence the presence of neurotransmitters at early stages
401 of development is established. Some other studies have shown significant involvement of
402 neurotransmitter – receptor interaction. Acetylcholine and nicotine induced hyperactive STC in 28 hpf
403 embryos and this is considered to be a result of activation of nicotinic acetylcholine receptors (nAChRs)
404 (Thomas et al. 2009). STC response was also abolished (hypoactivity) in a sodium channel knockdown
405 mutant in 24 hpf embryos (Chen et al. 2008). Spasmodic STC behavior and later on paralysis was observed
406 in an acetylcholinesterase (AChE) knockdown mutation in 27 hpf embryos and this could be due to the
407 over-excitation of the acetylcholine receptors by undegraded acetylcholine (Behra et al. 2002). Moreover,

embryonic response was abolished by cholinergic blockers - bungarotoxin and d-tubocurarine in 28 hpf embryos (Grunwald et al. 1988; Saint-Amant and Drapeau 1998). These results suggest that both electrical and chemical induced synapses at least play a part in mediating the STC response and hence, the STC endpoint is able to reveal effects of neuroactive chemicals on the synapses at an early zebrafish embryo age of 24 hpf.

#### 4.1.2 **Endpoint measurement -** Correct and accurate measurement of the endpoint

Measurement of the STC can be conducted by manually counting the coiling frequency or by analyzing videos with an automated workflow in KNIME®. Counts of the STC are normalized against control embryos to infer hyper- or hypoactivity. A detailed analysis was undertaken to compare the output of the automated analysis in KNIME® with manual counting. The results shown in Figure 3 indicates the accuracy of the automated analysis in KNIME®. Nevertheless, it is recommended to implement a correction protocol (as in section 4.3.1.4) to control for potential errors.

#### 4.1.3 **Dynamic range -** Determination of the extent of measurable change

The STC`s provide a dynamic range that allows to detect hyper- and hypoactivity effects relative to the control within the same assay. These effects can be quantified using hypothesis testing or dose-response modeling. The average STC count for untreated embryos can vary between 2-5 counts/min between experiments. Figure 5 shows the distribution of negative and solvent controls for all chemicals tested. An average STC count of 3.3 ± 0.85 /min was estimated for a pool of 94 replicates measured on different days. However, the trend of exposures are conserved. Therefore, we have normalized all data with respect to individual control from independent experiments and this could demonstrate reproducibility of the effects and allows for extensive concentration-response modelling.

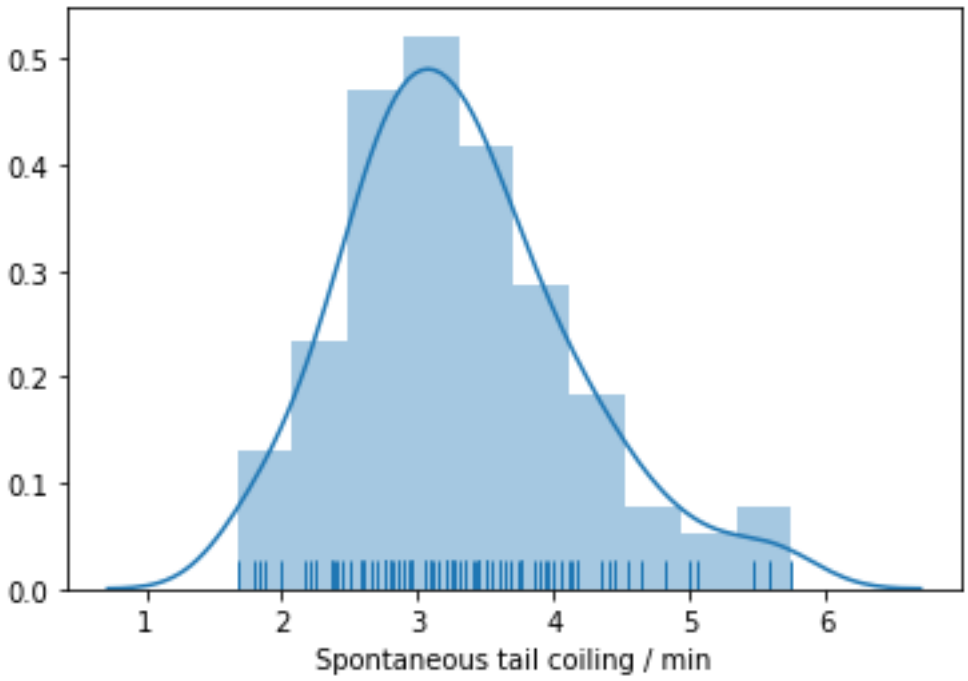



Figure 5: Histogram and density plot showing the distribution of 94 negative/solvent controls measured on different days.

**4.1.3 Parametric controls -** Assay parameters that predictably change the endpoint

**4.1.3.1 Effect of development stage**

To characterize the intrinsic behavior of a specific zebrafish strain, it is important to investigate the optimal STC response across different developmental stages or ages for that particular strain. Varying developmental stages from 21 hpf till 31 hpf showed an initial low response which then rapidly increased and peaked around 23 and 24 hpf, followed by a gradual decline until 31 hpf (Figure 1). To explore a high sensitivity of STC test, it is beneficial to measure during the peak response (23-25 hpf) in untreated embryos. Nevertheless, the full dynamic range and diagnostic capacity can be explored by measuring during a wider range of development stage (19-28 hpf). Similar to our result, Chen et al. (2012) reported control STC peak of 5 counts/min at 22, 23 and 24 hpf. Saint Amant and Drapeau (1998) characterized STC in dechorionated embryos and they did not only find significantly higher frequency (60/min), but peak

443 STC was observed at 19 hpf. We observed similar high frequency of ≈35/min when embryos were
444 dechorionated at 24 hpf (SI movie 1 and 2). Thomas et al. (2009) also reported peak STC at 19 hpf and
445 higher STC counts for dechorionated embryos. This discrepancy in STC counts for dechorionated embryos
446 could be due to the excessive stimulation as a result of direct contact with ionic media containing
447 potassium chloride (Thomas et al. 2009). To obtain robust toxicological information, it is recommended
448 to measure the STC of the fish strain at use over several time points to understand the intrinsic variability
449 of that strain.
450
451 **4.1.3.2 Effect of analysis duration**
452 Shorter analysis duration may allow to increase the throughput of STC tests. Therefore, we investigated
453 the effect of different analysis duration of 60, 30, 20 and 10 s. The results show a trend in which the STC
454 frequency slightly declined across the durations from 60 to 10 s (Figure 2). Even though the decline was
455 not statistical significant, it could mean a loss of STC peak information when shorter durations are used.
456 Raftery et al. (2014) utilized lower duration of 6 seconds and they reported that lower sensitivity observed
457 could be due to short duration. Shorter durations could be problematic especially for hypoactivity effects
458 in which an embryo could give only one peak which could occur at any time-point within a duration of 60
459 s. In such cases, a 60 s duration may be more robust to capture the STC response. Nevertheless shorter
460 durations of 30 and 20 s also appear to be mildly robust and could be used within a miniaturized setup.
461
462 **4.1.3.3 Effect of acclimation, sample size and rearing conditions**
463 Some experimental parameters did not seem to influence the STC response. For example, acclimation
464 time did not cause any change in STC counts within a duration of 30 minutes, even though the
465 temperature declined from incubation temperature of 28 °C to room temperature of 22.8 °C (SI Table S3).
466 Vliet et al. (2017) also found no effect of acclimation temperature on STC response when embryos were

acclimatized for 1 h at different temperatures. However, Saint Amant and Drapeau (1998) reported 40 % decline in STC after acclimatization to room temperature. They did not state the duration of acclimation and a confounding effect of developmental stage or the use of dechorionated embryos could be responsible for their observed decline in STC response. Nevertheless, we implemented an acclimation period of 30 min before measurement in our STC protocol. Manipulation of sample size by reducing number of embryos in a dish from 20 to 10 and increasing number of replicates from 3 to 5 did not seem to affect the variability of the STC (SI Table S3). Additionally, mean of 10 embryos appear to have similar STC response with mean of 20 embryos and therefore, 10 embryos could be used within a miniaturized setup or when lower exposure volume is required. Studies on rearing conditions show that group exposure conditions do not cause contagious stimulation of the STC due to movement of neighboring embryos (SI Figure S1). However, older embryos raised in groups showed a higher locomotor activity than those raised individually after the first 5 days of development (Zellner et al. 2011).

#### 4.3.1.4 Effect of image analysis parameters

To determine the optimal parameters for automated video-based STC analysis consistent with manual STC counting, we investigated the influence of threshold (thrs) and smoothing-parameter (spar) in peak detection analysis. Results show that both factors are equally influential such that an increase in one parameter needs to be balanced by the decrease in the other to obtain results consistent with manual counts. This is obvious because an increase in the smoothing-parameter will reduce the signal and a decrease in threshold will capture a reduced signal. Balance ratio of smoothing-parameter to threshold of 40 (0.1/0.0025), 50 (0.1/0.002) and 57 (0.2/0.0035) revealed similar in comparison to manual STC counts for untreated zebrafish embryos. It is important to note that some other confounding factors can influence the STC peak analysis. For example, uncontrolled events such as strong signal from movement

of whole embryo, unstable videos with background changes in pixels, many weak peaks close to the threshold and inaccurate accountability of fast multiple peaks may influence the STC response.

The use of 0.003thrs/0.1spar parameterization can handle some of these challenges. To ensure high quality STC data, it is also recommended to re-check the video and peaks for these potential errors and correct them accordingly in the initial setup. For example, our recommended KNIME® parameter produced 10% deviation from the true count in one of the independent replicates because of the strong effects of moving embryos. In such cases, manual correction will be more effective than changing the KNIME® parameters. A possible correction workflow can be: 1. Check for moving embryos 2. Visually inspect the peaks for errors. Irregular shaped and wide peaks are suspects 3. Manually count problematic embryos or peak areas.

**4.1.4 Response characterization -** Level of change determined to be an effect

The STC response which is considered to be a significant effect can be characterized using hypothesis testing or fitting a dose response model for ECx estimation. A response in hypothesis testing is defined as probability value below the threshold of 0.05. In this study, we used dose-response modeling to estimate $EC_{10}$ and $EC_{50}$ responses. This method was capable of accurately characterizing hyper- and hypoactivity responses in the STC test. In this study we did not consider or characterize the amplitude of STC or differentiate between strong and weak STC response.

**4.1.5 Concentration range** - Methods must be designed to allow determination of concentration-response

It is generally recommended to test a minimum of 5 concentrations to enable concentration-response modeling (OECD 236; Crofton et al. 2011). The STC test as devised in this study allows the convenient assessment of 15-20 dishes within a duration of ≈30 minutes by a single person. STC assessment within a

30 minutes time-frame reduces possible influences of changes in developmental stage on the STC response. In order to detect an STC effect, it is also essential that the concentration range covers the effective range of the chemical. Hence, no fixed concentration range should be applied. In contrast concentration ranges need to be adjusted for individual chemicals. For example, carbamazepine was only effective in the STC test after extending the concentration range from 0 – 80 µM to 0- 500 µM. Crofton et al. (2011) recommends 5 logs below the solubility limit of the chemical and we recommend to use, depending on the data available, maximum lethal concentrations of LC50/2 or LC10 as starting concentrations to avoid unspecific sublethal effects. Additionally, conducting an initial range-finding test may allow to consider lab-specific factors such as zebrafish strain and rearing conditions.

**4.1.6 Endpoint selectivity** - Discrimination of the endpoint of concern from non-specific outcomes

It is possible to assess non-specific outcomes such as developmental malformations during STC measurement. The effects on STC should be compared to effect concentrations for malformations or lethality in order to estimate the specificity of the effects. This ensures that observed behavior effects are not driven by morphological effects since malformed embryos could show hypoactivity (Padilla et al. 2011). It is worthy to note that chemicals causing hyperactivity such as organophosphates, may induce hypoactivity at high concentrations in non-deformed embryos. This could be due to over-excitation of the neuron cell leading to axonal defects or paralysis and this does not necessarily lead to observable phenotypes (Behra et al 2002; Stehr et al 2006, Piña-Crespo et al 2014). This biphasic response could be accounted for by testing an extensive concentration range covering both the hypo- and hyperactivity effects.

**4.1.7 Endpoint selective controls -** Chemicals known to reliably and consistently alter the endpoint at a mechanistic level

Abamectin and chlorpyrifos were identified as hypo- and hyperactivity controls respectively, while diuron and pyraclostrobin could represent suitable negative controls in the STC test. Abamectin consistently caused hypoactivity at an $EC_{50}$ of 0.055µM. Hypoactivity effects (LOEC) were also found for abamectin by Raftery et al. (2014) [3.1µM], Raftery et al. (2015) [0.25µM], Weichert et al. (2017) [0.72µM] and Vliet et al. (2017) [1.56µM]. The variation in hypoactivity effect concentrations for abamectin could be due to the use of hypothesis testing rather than dose-response modeling used in this study. Hyperactivity was also recorded for chlorpyrifos at an $EC_{50}$ of 1.85µM and this was consistent with the effects (LOEC) of Watson et al. (2014) [1µM] and Selderslaghs et al. (2010) [1.8µM]. The reproducibility of chlorpyrifos and abamectin, as demonstrated by comparing our studies to literature studies indicates the usability of these chemicals as positive controls in the STC test. However, a mechanistic level investigation is still required to verify how these chemicals alter the endpoint. Diuron did not induce any effect within the concentration range tested (1 - 8µM). However, diuron caused hypoactivity in another STC test at 16.3µM (Velki et al. 2017). This same concentration could not be assessed in the present study because it caused 100% lethality. Velki et al. (2017) did not only report hypoactivity at 16.3µM, but also incomplete tail coiling which could represent unspecific effects due to overt toxicity.

**4.1.8 Training set of chemicals** - Proof-of-concept that the test method can rapidly and efficiently screen moderate numbers of chemicals

The STC test as devised in this study for MoA identification takes approximately 2mins for measuring a single glass dish. This means a single chemical with 5 concentrations and 2 replicates will last approximately 20 mins. The required time can be reduced for rapid screening of chemicals in which lower number of concentrations and replicates are used. Furthermore, high resolution cameras and well plates can be applied in screenings to achieve a higher throughput. A total of 18 chemicals were tested in this study to evaluate the capability of the STC test to detect neuroactive substances. The chemicals were

558 classified based on their known mode of action to be hyperactive, hypoactive and not-active. Hyperactive
559 chemicals are expected to activate neuronal synapse while hypoactive ones are expected to inhibit
560 neuronal signal transduction, thereby causing increase and decrease in the STC respectively. Seven of the
561 exposed chemicals were expected to cause hyperactivity. The STC test detected hyperactivity for
562 chlorpyrifos, chlorpyrifos-oxon, paraoxon-methyl and diazinon with sensitivity ratios ($LC_{50}/EC_{50}$) of 2.9,
563 4.7, 55.7 and 3.7 respective to their 48 or 96h LC50 (Table 1). The hyperactivity effect of these substances
564 could be related to their proven capacity to inhibit acetylcholinesterase in zebrafish embryos (Kais et al.
565 2015; Küster 2005; Yang et al. 2011; Yen et al. 2011). This was revealed in the fact that chlorpyrifos was
566 about 6 times less toxic than chlorpyrifos-oxon which is the readily potent form to inhibit
567 acetylcholinesterase. Additionally, chlorpyrifos-oxon induced a biphasic effect i.e hyperactivity at low
568 concentrations and hypoactivity at higher concentration of 25 µM which could be an indication of axonal
569 deformation or over-excitation of nerve cells resulting in paralysis (Behra et al 2002; Ogungbemi et al.
570 2019). Paraoxon-methyl also induced sublethal effects (incomplete tail coiling and reduced-resorption of
571 yolk sac) at high concentration of 100 µM which could be indications of developmental delay (SI Figure
572 S3). Teixidó et al. (2013) also found developmental delay (reduced head-trunk angle and tail length) for
573 embryos exposed from 48-52 hpf to 20 µM paraoxon. Despite that hyperactivity has been reported for
574 aldicarb (Kokel et al. 2010) and nicotine (Leuthold et al. 2019; Thomas et al. 2009) in short exposure
575 behavior tests, both chemicals showed only a subtle and highly variable hyperactivity in the present study
576 (Figure 4). This may be attributed to low hydrophobicity (Log Kow of 1.2) which may lead to quick
577 attainment of steady state (Kühnert et al. 2013) and hence a relatively long exposure of 24 h could lead
578 to degradation/detoxification or a desensitization effect of these compounds. In particular, 30 µM
579 nicotine was found to reach steady state in 10 min for 23 hpf embryos. This then desensitized the nicotinic
580 acetylcholine receptors even after a 2 h depuration (Thomas et al. 2009). To further investigate this
581 possible desensitization of nicotine, we conducted an additional short duration exposure (20 mins) for

582 nicotine. Similar to the study by Thomas et al. (2009), we found a clear hyperactivity for nicotine at
583 different concentrations (10, 20, 30, 40 μM) which became minimal in a long duration exposure (SI Figure
584 S4). This result suggests short duration tests could be implemented as a second-tier or alongside long
585 duration tests to improve the diagnostic capacity of the STC, especially for substances with fast uptake
586 kinetics. Imidacloprid up to 2000 μM did not induce effect in the STC test. Despite imidacloprid has been
587 thought to be selective to insect nicotinic acetycholine receptors (nAChRs), some studies have reported
588 effects of imidacloprid on locomotor activity of 5dpf zebrafish (Leuthold et al 2019; Crosby et al 2015).
589 Absence of effect of imidacloprid in the present study may be due to specific effect of imidacloprid on the
590 brain nAChRs rather than the neuromuscular receptors which the STC measures.

591

592 Hypoactivity was detected for all four chemicals; abamectin, carbamazepine, diazepam and propafenone
593 with sensitivity ratios of 12.7, 0.97, 8.1 and 2.56 respective to their 48 h LC50 or baseline toxicity (Table
594 1). The hypoactivity effect of these substances could be related to their proven capacity to inhibit neuronal
595 synapses by activating GABA gated chloride channels or blocking sodium channels (Söderpalm 2002).
596 Carbamazepine induced hypoactivity ($EC_{50}$ = 195 μM) in Weichert et al. (2017) and this was only 1.4 fold
597 lower than $EC_{50}$ of 271 μM obtained in the present study. Both values are in the same range as the 48 h
598 $LC_{50}$ (263 μM) and this low sensitivity of the STC for carbamazepine could be due to similar issues related
599 to low hydrophobicity and quick attainment of steady state as discussed for nicotine above (Halbach et al
600 2020).

601 In search for non-active chemicals, we exposed 6 chemicals with unknown or no reported neuroactive
602 mode of action. The ideal negative controls are chemicals that induce effect on other biological systems,
603 but are not expected to disrupt the nervous system (Aschner et al 2017). Birke and Scholz (2019) classified
604 aniline and pyraclostrobin to be narcotic substances based on their toxic ratio (defined as the ratio of a

chemical's $LC_{50}$ estimated from a QSAR for baseline toxicity and the experimental $LC_{50}$) value of 5.4 and 3.1 respectively. Other negative substances were selected based on unknown neurotoxic MoA. Only pyraclostrobin, daunorubicin-hydrochloride and diuron did not cause STC effect (SI Figure S5). No STC effect for pyraclostrobin has already been reported up to 0.76 µM (Raftery et al 2014). Interestingly, the STC test detected hyperactivity for hexaconazole, aniline and 3,4-dichloroaniline with sensitivity ratios of 16, 2.3 and 2.6 respectively to their 24 or 48 hpf $LC_{50}$ (Table 1). We consider hyperactivity to represent a specific effect on STC since unspecific secondary effects caused by cytotoxicity and/or malformation may rather result in hypoactivity. In fact, an hexaconazole containing product has been reported to cause neurotoxic effects such as trembling, jittering and shaking in a poisoned human (David et al. 2008) and hexaconazole is classified as neurotoxic to the human nervous system (Grandjean and Landrigan 2014). Similar to our findings, hexaconazole also induced hyperactivity in the zebrafish embryo photomotor response test (Reif et al. 2016). Hexaconazole also decreased thyroxine (T4) levels while increasing triiodothyronine (T3) in 120 hpf zebrafish embryos (Yu et al. 2013). Hyperactivity effects of T3 and T4 on light/dark induced locomotor response of 120 hpf zebrafish embryo have also been reported (Walter et al. 2019). Subsequently, the hyperactivity induced in the STC test by hexaconazole may be associated to thyroid hormone disruption impacting the proper development of the motor neurons. An alternative hypothesis is hexaconazole may induce hyperactivity by blocking GABA receptors similar to its structurally related pentylenetetrazole (Squires et al. 1984). Aniline is classified as neurotoxic in the pesticide properties database and tremor manifestations was associated to aniline exposure (National research council 2008). Interestingly, a commonly used solvent, DMSO, also induced hyperactivity in the STC test despite that it has been listed as a potential negative control for developmental neurotoxicity (Aschner et al 2017). Following from these results, we can consider the STC test valuable to indicate indirect effects on the nervous system.

628 **4.1.9 Specificity and sensitivity** - Analysis to determine ability to correctly differentiate active and non-
629 active chemicals

630 The STC test was able to accurately detect 8 out of 11 neuroactive substances, amounting to 73%
631 sensitivity. However, the results from this study are too few to reliably estimate specificity and sensitivity.
632 Moreover, it is difficult to estimate the specificity of the test because substances which do not have a
633 known neuroactive mode of action may have unknown or indirect neuroactive side-effects like in the case
634 of hexaconazole or aniline. Similarly, chemicals which show neurotoxic effect may induce this via non
635 neural organs or receptors.

636 **4.1.10 High throughput** - Test system and endpoint should be amenable to automation

637 The STC test can be considered to be a mid-high throughput test because of its short test duration of 24
638 h compared to other behavior tests, short video acquisition duration and possible automated workflows
639 for estimating the STC frequency. It may be further optimized to comply with analysis in 96well plates
640 which could further improve throughput. However, utilizing plastic 96well plates may compromise effect
641 concentrations due to sorption of lipophilic compounds to plastic wells. **4.1.11 Documentation -** Full
642 and published documentation of the test method Resources

643 Full documentation of the STC test as used in the current study can be found within the method section
644 and within the complementary method paper associated to this study.

645 **4.1.12 Transferability -** Resources for use should be available for any laboratory

646 The required resources for easy implementation of the STC test are accessible and widely available. The
647 test organism, zebrafish, is a model organism and can be easily reared in indoor aquaria. Moreover, the
648 eggs obtained from the adults can be synchronized by cell stage. The glass crystallization exposure dish
649 can be readily purchased. The assessment tools; microscope and camera are regularly used resources in

most biology laboratories and can be easily purchased and set up. Most especially, we provide a workflow for automated STC counting within the KNIME® platform. This workflow is freely available by searching for "spontaneous tail coilings detection in zebrafish" on https://hub.knime.com/ and can be easily implemented by following basic instructions outlined in the associated method paper or by watching this video - https://youtu.be/wgJN71zTvRw. This means that laboratories that cannot afford commercially available software can still maximize the capacity of the STC test.

# 5.0 Conclusion

In this study, we optimized the STC test and investigated the effect of 18 chemicals with different MoA. We show that developmental stage and analysis duration can influence the STC response. Based on this, we selected 24-25 hpf and 1 min as the optimal developmental stage and analysis duration for testing. Other parameters such as acclimation duration (within 30 mins), sample size and rearing conditions had no observable impact. Consequently, we selected a sample size of 20 embryos, group rearing condition and acclimatized the sample at room temperature for 30 min before analysis. Apart from a MATLAB® tool (González-Fraga et al. 2019) which still requires a paid version of MATLAB®, our KNIME® workflow is the only available freeware for STC analysis. The optimized STC test showed high sensitivity by detecting 8 out of 11 neuroactive substances at concentrations below their acute or baseline lethality. Interestingly, the STC test could also detect effects for substances with unknown neuroactive MoA which indicates possible neuroactive side effects or unknown mechanisms of action that impact on the STC. Two of the chemicals tested in this study (chlorpyrifos and nicotine) are classified as reference compounds for developmental neurotoxicity (Aschner et al 2017). In conclusion, we show the high potential of the STC test to screen developmental neurotoxicity for hazard assessment and for effect-based environmental monitoring.

Therefore, a desired next step will be to harmonize and validate the STC test for prospective and diagnostic testing.

## Acknowledgement

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